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An Insulin Analogue Possessing Higher in Vitro Biological Activity than Receptor Binding Affinity. [21-Proline-B]insulin†

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ABSTRACT: To study the effect of an increase in the potential for β-turn formation of the B^{20}–B^{23} segment of the B-chain moiety on the biological behavior of insulin, the [21-proline-B]insulin ([Pro^{21}-B]insulin) was synthesized. The in vitro biological activity and the receptor binding affinity of this analogue were compared with that of insulin. In stimulating labeled glucose incorporation into lipids in rat fat cells, the analogue displayed 33.2% potency relative to insulin; receptor binding affinity for the analogue was 15.9% in rat liver membranes and 17.8% in isolated fat cells. [Pro^{21}-B]insulin is thus the first example of a modified insulin for which the biological activity exceeds the receptor binding potency. The secondary structure of this analogue was investigated by circular dichroism studies. Although no significant differences in the conformation of monomeric insulin and analogue could be discerned, their difference in behavior with respect to dimerization and biological properties indicates that these forms are not equivalent. We suggest that the intrinsic activity of receptor-bound [Pro^{21}-B]insulin is greater than that of insulin, although the receptor displays greater affinity for insulin than for the analogue. We consider a model for the interaction between insulin and its receptor that accommodates our findings.

X-ray analysis of the insulin molecule indicates that the B chain segment -Gly-Glu-Arg-Gly-, occupying positions B^{20}–B^{23}, forms a turn within the molecule so that residues B^{24}–B^{30} lie antiparallel and against the B^{9}–B^{19} α-helical segment (Blundell et al., 1972). This arrangement generates interactions which have been postulated to be important in the maintenance of the structure and hence the activity of insulin (Blundell et al., 1972).

By the Chou–Fasman method for prediction of secondary structure (Chou & Fasman, 1978), the sequence B^{20}–B^{23} has a relatively low potential for β-turn formation with $(P_t) = 1.2$ and $p_t = 9.2 \times 10^{-5}$, slightly exeeding the minimum values of these parameters for β-turn prediction [$(P_t) > 1$ and $p_t > 7.5 \times 10^{-5}$]. It was therefore of interest to investigate what effect an increase in the potential for β-turn formation of the B^{20}–B^{23} segment would have on the biological activity of insulin. Replacement of Glu found in the second position of the above-mentioned tetrapeptide sequence by Pro would considerably increase its potential for β-turn formation with $(P_t) = 1.4$ and $p_t = 4.6 \times 10^{-4}$. The present paper describes the synthesis and biological evaluation of [Pro^{21}-B]insulin in which the glutamic acid residue found at the B^{21} position in the

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natural hormone is replaced by a proline residue.

Experimental Procedures

Details of the synthetic and analytical procedures used are given in a previous publication (Schwartz et al., 1981). Preswollen microgranular CM[1]-cellulose (Whatman CM52) was used in this study. The washing of the resin and the preparation of the columns and of the buffers used were described previously (Katsoyannis et al., 1967a,b). Isoelectric focusing on thin-layer plates was carried out as described previously (Schwartz et al., 1981). Sodium tetrathionate was prepared as described by Gilman et al. (1946). [3-^{3}H]Glucose (11.5 Ci/mmol), porcine ^{125}I-insulin (75–90 μCi/μg), and Aquasol-2 scintillation fluid were purchased from New England Nuclear. Filtron-X and Soluscint-O scintillation fluids were products of National Diagnostics Co., Somerville, NJ. Cellulose filters (type EGWP) were obtained from Millipore Corp., Bedford, MA. Fine mesh silk (Buehler Polishing Cloth, catalog no. 40-7408) was obtained from VWR Scientific Inc. Collagenase type II was purchased from Worthington. Fatty acid free albumin and crystalline bovine insulin were products of Sigma, and fraction V bovine serum albumin was from Schwarz/Mann, Orangeburg, NY.

^{125}I-Insulin Binding: Liver Plasma Membranes. Plasma membrane enriched fractions were prepared from the livers of fasted rats essentially as previously described (Horvat et al., 1975) and were assayed for ^{125}I-insulin binding in the presence of varying concentrations of bovine insulin or analogue in triplicate (Burke et al., 1980). Unbound label was removed by filtration on cellulose acetate filters, and nonspecific binding, defined as label remaining on the filter when membranes were incubated in the presence of 60 μg/mL unlabeled insulin, was subtracted from all values. Nonspecific binding was generally about 10% of the radioactivity bound in the absence of unlabeled competitor. Relative potency is expressed as

$$\frac{\text{concentration of insulin (ng/mL)}}{\text{concentration of analogue (ng/mL)}} \times 100$$

required to displace 50% of specifically bound ^{125}I-insulin.

^{125}I-Insulin Binding: Rat Adipocytes. The behavior of insulin and analogue in competition with ^{125}I-insulin for insulin receptors of adipocytes was examined as described previously (Burke et al., 1980). Unbound label was separated from cells by centrifugation through dinonyl phthalate. Nonspecific binding was about 15% of the radioactivity bound in the absence of unlabeled competitor. Relative potency is expressed as described for liver plasma membrane receptor binding. All incubations were performed in triplicate.

Lipogenesis. Rat adipocytes, prepared by incubation of fat pads with crude collagenase (Rodbell, 1964), were used to evaluate the ability of insulin and analogue to stimulate the conversion of [3-^{3}H]glucose in triplicate incubations into material extractable into Soluscint-O as previously described (Burke et al., 1980). Lipogenesis incubations were stopped by the addition of 0.2 mL of 5 N H_2SO_4, and 0.2 mL of corn oil was added to aid in the extraction of labeled lipids. Stimulation of 0 and 100% represents conversion of labeled glucose in the absence and presence, respectively, of 5.46 ng/mL bovine insulin. Relative potency is expressed as

$$\frac{\text{concentration of insulin (ng/mL)}}{\text{concentration of analogue (ng/mL)}} \times 100$$

required to achieve 50% of the maximum stimulation of conversion of [3-^{3}H]glucose into organic-extractable material. Maximum stimulation by insulin was 5–10-fold over the basal activity.

Circular Dichroism (CD). A JASCO J-500A automatic recording spectrophotometer equipped with a data processor was used for CD measurements of the [Pro^{21}-B] analogue and zinc-free insulin. Spectra were taken at 26° C, under nitrogen, in 0.001 M HCl (pH 3.2) with protein concentrations of ca. 0.01–0.10 mg/mL. Cylindrical quartz cells with optical path lengths of 0.10–2.00 cm were used. Each sample was scanned 4 or 8 times, and an average spectrum was obtained by using the data processor and correcting for the base line. Mean residue ellipticity ($[\theta]^{mrw}$) was calculated from

$$[\theta]^{mrw} = \theta\bar{M}/(cl)$$

where θ is the observed ellipticity, $\bar{M}$ is the mean residue molecular weight (mrw) calculated to be 112 for insulin and the analogue, c is the protein concentration in milligrams per milliliter, and l is the optical path length in centimeters.

General Aspects of Synthesis of [Pro^{21}-B]insulin. The synthesis of this analogue was accomplished by the combination of the sulfhydryl form of bovine insulin A chain with the S-sulfonated form of human [Pro^{21}]B chain, according to the procedure described previously (Schwartz & Katsoyannis, 1976). The sulfhydryl form of bovine A chain was obtained by reduction with 2-mercaptoethanol of the S-sulfonated derivative of that chain, prepared by oxidative sulfitoylsis of bovine insulin and separation of the resulting S-sulfonated A and B chains by column chromatography (Katsoyannis et al., 1967a). The synthesis of the S-sulfonated human [Pro^{21}]B chain was accomplished by procedures similar to those employed for the synthesis of human B chain (Schwartz & Katsoyannis, 1973b). It involved the synthesis of the protected triacontapeptide (IVa) embodying the entire amino acid sequence of the human [Pro^{21}]B chain, removal of the protecting groups upon treatment with liquid hydrogen fluoride, and conversion of the resulting thiol derivative into the S-sulfonated form (IV) by oxidative sulfitolysis. The synthesis of the protected triacontapeptide was carried out by the classical methods of peptide synthesis. It involved the condensation of the C-terminal decapeptide (I) (sequence B^{21}–B^{30}) with the adjacent hexapeptide (sequence B^{15}–B^{20}) (Schwartz & Katsoyannis, 1973a) to produce the C-terminal hexadecapeptide (II) (sequence B^{15}–B^{30}) which in turn was condensed with the adjacent hexapeptide (sequence B^{9}–B^{14}) (Schwartz & Katsoyannis, 1973b) to yield the C-terminal docosapeptide (III) (sequence B^{9}–B^{30}). The latter fragment was selectively deblocked at the α-amino end and coupled with the N-terminal octapeptide (sequence B^{1}–B^{8}) (Schwartz & Katsoyannis, 1973b) to produce the protected triacontapeptide IVa.

N-(tert-Butoxycarbonyl)-L-prolyl-N$^{\omega}$-nitro-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-L-threonyl-L-prolyl-N$^{\epsilon}$-(benzyloxycarbonyl)-L-lysyl-O-benzyl-L-threonine Benzyl Ester (I). A solution of *N*-(*tert*-butoxycarbonyl)-N^{ω}-nitro-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-*O*-benzyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-(benzyloxycarbonyl)-L-lysyl-*O*-benzyl-L-threonine benzyl ester (Schwartz & Katsoyannis, 1973a) (1.4 g) in a mixture of trifluoroacetic acid–acetic acid, 7:3 (v/v) (Bodanszky et al., 1978) (20 mL), was stored at room temperature for 30 min and then poured into cold ether (300 mL). After 3 h at 0 °C the precipitated product was filtered off, washed with ether, and dried over

[1] Abbreviations: CM, carboxylmethyl; DMF, dimethylformamide; Me_2SO, dimethyl sulfoxide; Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride.

KOH in vacuo. This product, along with *N*-methylmorpholine (0.1 mL), was added to the carboxyl component which was preactivated as follows. To a solution of *N*-(*tert*-butoxycarbonyl)-L-proline (428 mg) in DMF (10 mL) were added 1-hydroxybenzotriazole (300 mg) and *N,N'*-dicyclohexylcarbodiimide (400 mg) followed after stirring for 1 h at room temperature by the deblocked nonapeptide prepared as just described. After 24 h at room temperature, the reaction mixture was diluted with 2-propanol (200 mL) and ether (100 mL). The precipitated protected decapeptide was filtered off and reprecipitated from 95% ethanol and from a solution in DMF by the addition of acetone: yield 1.1 g (79%); mp 188–190 °C; $[\alpha]^{25}_D$ −34.9° (*c* 1, DMF). Anal. Calcd for $C_{93}H_{115}N_{15}O_{20}\cdot H_2O$: C, 62.7; H, 6.62; N, 11.8. Found: C, 62.1; H, 6.71; N, 11.6. This peptide was homogeneous on thin-layer chromatography in the system chloroform–methanol–water (45:10:1 v/v).

N-(*tert-Butoxycarbonyl*)-L-*leucyl-O-benzyl*-L-*tyrosyl*-L-*leucyl*-L-*valyl-S*-(*diphenylmethyl*)-L-*cysteinylglycyl*-L-*prolyl-N*$^{\omega}$-*nitro*-L-*arginylglycyl*-L-*phenylalanyl*-L-*phenylalanyl-O-benzyl*-L-*tyrosyl*-L-*threonyl*-L-*prolyl-N*$^{\epsilon}$-(*benzyloxycarbonyl*)-L-*lysyl*-L-*threonine Benzyl Ester* (*II*). Compound I (709 mg) was treated with a mixture of trifluoroacetic acid–acetic acid (7:3) (10 mL). After 1 h the solution was diluted with cold ether (100 mL) and stored in the cold (0 °C) for 1 h. The precipitated decapeptide trifluoroacetate salt was filtered off, washed with ether, and dried. A solution of this product in DMF (5 mL) containing *N*-methylmorpholine (0.05 mL) was added to the hexapeptide carboxyl component, activated as follows. A solution of *N*-(*tert*-butoxycarbonyl)-L-leucyl-*O*-benzyl-L-tyrosyl-L-leucyl-L-valyl-*S*-(diphenylmethyl)-L-cysteinylglycine (Schwartz & Katsoyannis, 1973a) (1 g) in a mixture of DMF (6 mL) and Me_2SO (2 mL) was cooled to 4 °C and 1-hydroxybenzotriazole (160 mg) and *N,N'*-dicyclohexylcarbodiimide (200 mg) were added. After stirring for 30 min at 4 °C and 1 h at room temperature, the mixture was added to the solution of the deblocked decapeptide prepared as described previously. The reaction mixture was stirred for 24 h and then diluted with a saturated aqueous NaCl solution (500 mL) containing 1 N NH_4OH (20 mL). The precipitated hexadecapeptide derivative was isolated by centrifugation, washed with water until neutral, dried, and reprecipitated from a solution in DMF by the addition of 2-propanol: yield 1.1 g (97%). Amino acid analysis of an acid hydrolysate showed the following compositon expressed in molar ratios: $Lys_{0.8}Arg_{0.7}Thr_{1.6}Pro_{1.7}Gly_{2.0}Val_{1.1}Leu_{2.0}Tyr_{1.5}Phe_{1.7}{}^1/_2Cys_{0.6}$.

N-(*tert-Butoxycarbonyl*)-*O-benzyl*-L-*seryl-N*^{im}-*tosyl*-L-*histidyl*-L-*leucyl*-L-*valyl*-γ-*benzyl*-L-*glutamyl*-L-*alanyl*-L-*leucyl-O-benzyl*-L-*tyrosyl*-L-*leucyl*-L-*valyl-S*-(*diphenylmethyl*)-L-*cysteinylglycyl*-L-*prolyl-N*$^{\omega}$-*nitro*-L-*arginylglycyl*-L-*phenylalanyl*-L-*phenylalanyl-O-benzyl*-L-*tyrosyl*-L-*threonyl*-L-*prolyl-N*$^{\epsilon}$-(*benzyloxycarbonyl*)-L-*lysyl-O-benzyl*-L-*threonine Benzyl Ester* (*III*). The protected hexadecapeptide II (700 mg) was deblocked with trifluoroacetic acid–acetic acid mixture (7:3) (10 mL) as described previously. A solution of the resulting trifluoroacetate salt in *N*-methylpyrrolidinone (10 mL) containing triethylamine (0.1 mL) was diluted with ether (100 mL), and the precipitated free base was filtered off, washed with ether, and dried. To a solution of this product in DMF (6 mL), cooled to 4 °C, was added *N*-(*tert*-butoxycarbonyl)-*O*-benzyl-L-seryl-*N*^{im}-tosyl-L-histidyl-L-leucyl-L-valyl-γ-benzyl-L-glutamyl-L-alanine (Schwartz & Katsoyannis, 1973b) (484 mg), followed by 1-hydroxybenzotriazole (80 mg) and *N,N'*-dicyclohexylcarbodiimide (100 mg). After stirring at 4 °C for 48 h, the reaction mixture was diluted with 2-propanol (100 mL). The precipitated docosapeptide derivative was collected by filtration and reprecipitated from a solution in DMF by the addition of water: yield 420 mg (50%). Amino acid analysis of an acid hydrolysate gave the following composition expressed in molar ratios: $Lys_{0.9}His_{0.9}Arg_{0.7}Thr_{1.9}Ser_{0.9}Pro_{2.0}Glu_{0.9}Gly_{2.0}Ala_{1.0}{}^1/_2Cys_{0.7}Val_{1.7}Leu_{2.9}Tyr_{1.2}Phe_{1.7}$.

L-*Phenylalanyl*-L-*valyl*-L-*asparaginyl*-L-*glutaminyl*-L-*histidyl*-L-*leucyl-S-sulfo*-L-*cysteinylglycyl*-L-*seryl*-L-*histidyl*-L-*leucyl*-L-*valyl*-L-*glutamyl*-L-*alanyl*-L-*leucyl*-L-*tyrosyl*-L-*leucyl*-L-*valyl-S-sulfo*-L-*cysteinylglycyl*-L-*prolyl*-L-*arginylglycyl*-L-*phenylalanyl*-L-*phenylalanyl*-L-*tyrosyl*-L-*threonyl*-L-*prolyl*-L-*lysyl*-L-*threonine* (*Human Insulin* [*Pro*^{21}]*B Chain S-Sulfonate*) (*IV*). The protected docosapeptide III (400 mg) was deblocked with a mixture of trifluoroacetic acid–acetic acid (7:3) (5 mL) in the usual way. The resulting trifluoroacetic salt of the docosapeptide was converted to the free base as described in the synthesis of compound III. To a solution of the free base in DMF (5 mL) was added the octapeptide *N*-(*tert*-butoxycarbonyl)-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-L-histidyl-L-leucyl-*S*-(diphenylmethyl)-L-cysteinylglycine (Schwartz & Katsoyannis, 1973b) (500 mg). The mixture was stirred for 5 min, and to the resulting solution were added 1-hydroxybenzotriazole (90 mg) and *N,N'*-dicyclohexylcarbodiimide (100 mg). After 48 h the reaction mixture was poured into cold water (300 mL) containing 1 N NH_4OH (5 mL), and the precipitated protected triacontapeptide (IVa) was isolated by centrifugation, washed successively with water, 50% aqueous methanol, and methanol, and reprecipitated from a solution in DMF by the addition of methanol: yield 500 mg (90%). Amino acid analysis after acid hydrolysis gave the following composition expressed in molar ratios: $Lys_{0.9}His_{1.8}Arg_{0.8}Asp_{1.0}Thr_{1.7}Ser_{0.9}Pro_{1.8}Glu_{2.1}Gly_{3.0}Ala_{1.0}{}^1/_2Cys_{1.2}Val_{3.0}Leu_{3.9}Tyr_{1.8}Phe_{2.7}$.

This product was converted to the S-sulfonated [Pro^{21}]B chain by deblocking with liquid hydrogen fluoride followed by oxidative sulfitolysis by the procedure used in the synthesis of the S-sulfonated human insulin B chain (Schwartz & Katsoyannis, 1973b). In a typical experiment the protected triacontapeptide (200 mg) was treated with anhydrous liquid hydrogen fluoride (9 mL) containing anisole (1 mL) at 10 °C for 1 h. The hydrogen fluoride was then removed, and the residue was dried under high vacuum, over KOH, and triturated with ethyl acetate. This product was dissolved in 8 M guanidine hydrochloride (20 mL) and to this solution, adjusted to pH 8.9 with dilute NH_4OH or acetic acid (depending on the pH of the solution, were added sodium sulfite (1.0 g) and sodium tetrathionate (0.7 g). The mixture was stirred at room temperature for 3 h, placed in Spectrapor membrane tubing no. 3, dialyzed against four changes of distilled water (4 L each) at 4 °C for 24 h, and lyophilized.

For purification the lyophilized material was dissolved in 6 mL of urea–acetate buffer (0.04 M acetate–8 M urea, pH 4.0) and applied to a CM-cellulose column (4 × 10 cm) equilibrated and eluted with the same buffer. The preparation of column and buffer has been described previously (Katsoyannis et al., 1967a). The column effluent was monitored with an ISCO recording spectrophotometer (Model U-5A), and the elution pattern is shown in Figure 1. The eluate under the major peak (530–700 mL) was collected, desalted by dialysis (Spectrapor membrane no. 3) against four changes of distilled water (4 L each) for 24 h at 4 °C, and lyophilized to a white fluffy material; yield 40 mg. Amino acid analysis of the purified chain analogue after acid hydrolysis gave the

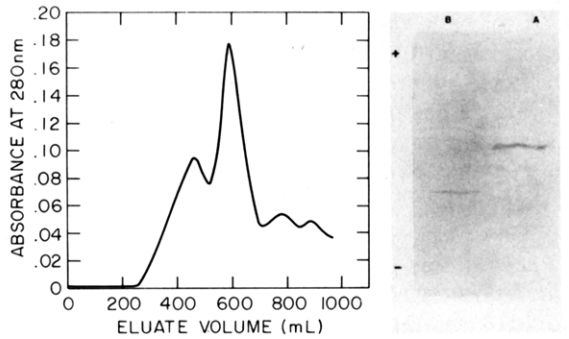

FIGURE 1: Chromatography of crude human [Pro^{21}]B chain *S*-sulfonate on a 4 × 60 cm CM-cellulose column with urea–acetate buffer (pH 4.0). The column effluent was monitored with an ISCO recording spectrophotometer. The purified chain (530–700 mL) was recovered after dialysis and lyophilization. (Inset) Paper print of thin-layer isoelectric focusing of natural bovine B chain *S*-sulfonate (A) and synthetic human [Pro^{21}]B chain *S*-sulfonate (B) in a 1:1 mixture of pH 3–10 and pH 4–6 ampholytes on a 20-cm separation distance. Focusing: constant power, 8 W for 4 h.

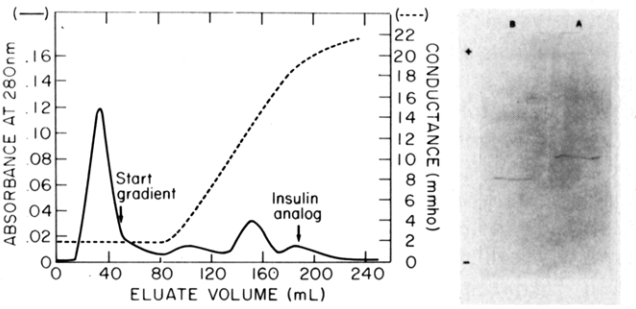

FIGURE 2: Chromatography of a combination mixture (see Experimental Procedures) of the thiol form of bovine A chain with the S-sulfonated form of human [Pro^{21}]B chain on a 0.9 × 23 cm CM-cellulose column with acetate buffer (Na^+, 0.024 M, pH 3.3) and an exponential NaCl gradient. The column effluent was monitored with an ISCO recording spectrophotometer and a conductivity meter (Radiometer, Copenhagen). The [Pro^{21}-B]insulin (170–230 mL of effluent) was recovered as the hydrochloride. (Inset) Paper print of thin-layer isoelectric focusing of natural bovine insulin (A) and synthetic [Pro^{21}-B]insulin (B) in a 1:1 mixture of pH 3–10 and pH 4–6 ampholytes on a 20-cm separation distance. Focusing: constant power, 8 W for 4 h.

following composition expressed in molar ratios, in good agreement with the theoretically expected values: $Lys_{0.9}His_{1.9}Arg_{0.9}Asp_{1.1}Thr_{1.8}Ser_{1.0}Pro_{2.0}Glu_{2.1}Gly_{3.0}Ala_{1.0}$-$^1/_2$-$Cys_{1.6}Val_{2.6}Leu_{3.7}Tyr_{1.6}Phe_{2.8}$. Theory: $Lys_{1.0}His_{2.0}$-$Arg_{1.0}Asp_{1.0}Thr_{2.0}Ser_{1.0}Pro_{2.0}Glu_{2.0}Gly_{3.0}Ala_{1.0}$$^1/_2$-$Cys_{2.0}$-$Val_{3.0}Leu_{4.0}Tyr_{2.0}Phe_{3.0}$. The synthetic chain was completely digested by aminopeptidase M. On thin-layer isoelectric focusing in a 1:1 mixture of pH 3–10 and pH 4–6 ampholytes, the S-sulfonated B chain analogue produced only one band closer to the cathode than the natural B chain *S*-sulfonate (Figure 1, inset).

Synthesis and Isolation of [Pro^{21}-B]insulin. The synthesis of this analogue was accomplished by the interaction of the sulfhydryl form of the A chain of bovine insulin with the S-sulfonated form of human [Pro^{21}]B chain by the procedure described previously (Schwartz & Katsoyannis, 1976). Briefly, a solution of 40 mg of bovine A chain *S*-sulfonate in 0.1 M Tris-HCl buffer (pH 8.3) was treated with 2-mercaptoethanol at 37 °C for 6–8 min under nitrogen. The mixture was then cooled to 4 °C and diluted with a mixture of acetic acid–2-propanol–ethyl acetate (1:30:50 v/v), and the precipitated thiol form of the A chain was isolated by centrifugation, washed with ethyl acetate and petroleum ether, and dried. This product was reacted with 20 mg of [Pro^{21}]B chain for 16 h at pH 10.6 and 4 °C. The reaction mixture was then treated as described previously (Katsoyannis et al., 1967b,c). Isolation of [Pro^{21}-B]insulin from the reaction mixture and purification were accomplished by chromatography on a CM-cellulose column (0.9 × 23 cm) with acetate buffer (Na^+, 0.024 M, pH 3.3) and an exponential NaCl gradient as described previously (Katsoyannis et al., 1967c). The chromatographic pattern, as monitored by an ISCO recording spectrophotometer and a conductivity meter (Radiometer, Copenhagen), is shown in Figure 2. The effluent containing the insulin analogue (170–230 mL) was processed as described previously (Katsoyannis et al., 1967c), and the purified [Pro^{21}-B]insulin was isolated via picrate as the hydrochloride (0.8 mg). Amino acid analysis of an acid hydrolysate of the analogue gave a composition in agreement with the expected values (Table I). Upon isoelectric focusing on thin-layer plates in a 1:1 mixture of pH 3–10 and pH 4–6 ampholytes, the [Pro^{21}-B]insulin focused into one band closer to the cathode than natural insulin (Figure 2, inset).

Biological Evaluation of [Pro^{21}-B]insulin. The displacement of ^{125}I-insulin from insulin receptors in rat liver plasma membranes and in isolated rat fat cells by unlabeled insulin

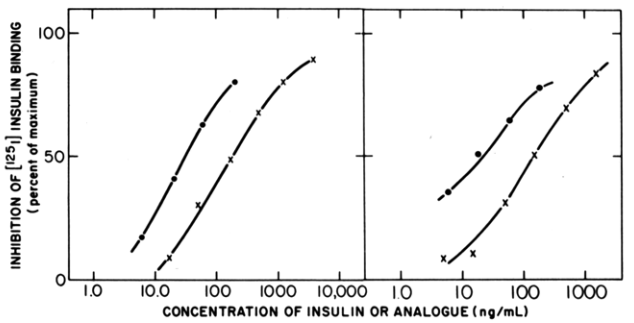

FIGURE 3: Effect of bovine insulin (●) and [Pro^{21}-B]insulin (×) on the binding of porcine ^{125}I-insulin to rat liver membranes (right) and to isolated fat cells (left). The inhibition of ^{125}I-insulin binding, expressed as percent of maximum, is plotted as a function of the concentration of unlabeled insulin and analogue. Data are from a typical experiment which was repeated 4 times for membranes and 3 times for fat cells.

Table I: Amino Acid Composition[a] of an Acid Hydrolysate of [Pro^{21}-B]insulin

amino acid	theory	found	amino acid	theory	found
Lys	1	0.9	Gly	4	3.9
His	2	1.9	Ala	2	1.9
Arg	1	0.8	$^1/_2$-Cys	6	3.4[b]
Asp	3	3.1	Val	5	4.4
Thr	2	1.8	Ile	1	0.6
Ser	3	3.0	Leu	6	6.0
Glu	6	6.1	Tyr	4	3.8
Pro	2	1.7	Phe	3	2.8

[a] Number of amino acid residues per molecule. [b] Uncorrected for destruction.

and by [Pro^{21}-B]insulin is depicted in Figure 3. Dose–response curves are essentially parallel, and the calculated relative potency of the analogue is 15.9 ± 5.2% in membrane assays and 17.8% in fat cells.

The ability of [Pro^{21}-B]insulin to stimulate lipogenesis in rat adipocytes is illustrated in Figure 4. The analogue is a full agonist reaching the same maximum activity as insulin, and the dose–response curves are parallel. In contrast to the receptor-affinity data, however, relative potency in this assay is calculated at 33.2 ± 5.0%, or at least twice the potency expected on the basis of receptor-binding experiments ($p < 0.005$ by Student's t test). Two separate preparations of the

Table II: Mean Residue Weight Ellipticity of [Pro^{21}-B]insulin and Bovine Insulin at Selected Wavelengths as a Function of Concentration

compound	concn (M)[a]	deg cm^2 dmol^{-1} (10^3) $+[\theta]_{193-195}$	$-[\theta]_{208}$	$-[\theta]_{220-222}$	$-[\theta]_{265-274}$
insulin	1.74×10^{-5}	20.0	13.3	9.7	150
insulin	7.00×10^{-6}	18.9	10.9	7.9	140
insulin	1.74×10^{-6}	15.3	10.4	7.0	112
[Pro^{21}-B]insulin	7.80×10^{-6}	11.2	9.5	7.0	116

[a] In 0.001 M HCl.

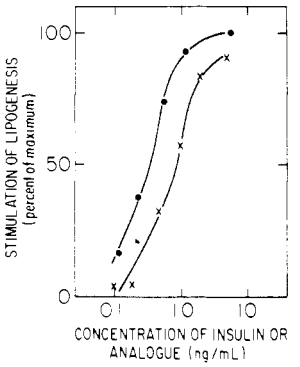

FIGURE 4: Effect of bovine insulin (●) and [Pro^{21}-B]insulin (×) on lipogenesis in isolated fat cells. The stimulation of lipogenesis, expressed as percent of maximum, is presented as a function of the concentration of insulin and analogue. Data are from a typical experiment which was repeated 4 times.

analogue displayed similar behavior. [Pro^{21}-B]insulin is the first example of an analogue displaying greater biological activity than receptor-binding affinity.

Circular Dichroism Studies. Figure 5 illustrates the near- and far-ultraviolet circular dichroic spectra of [Pro^{21}-B]insulin and zinc-free insulin. Under the same conditions, 0.001 N HCl and ca. 7×10^{-6} M concentrations, the analogue exhibited negative minima at 207.5, 220 (shallow inflection), and 270 nm and a maximum at 193 nm, all slightly shifted to shorter wave lengths from those observed with insulin: 195, 208, 222, and 272 nm. The molar ellipticities at the aforementioned maximum and minima of [Pro^{21}-B]insulin and three concentrations of zinc-free insulin are given in Table II.

Discussion

Until very recently, all biologically active insulin analogues prepared either by modification of the natural hormone (Blundell et al., 1972; Gliemann & Gammeltoft, 1974) or by chemical synthesis (Katsoyannis, 1981) exhibited the same ratio of relative potency for receptor binding affinity to relative in vitro biological activity. These analogues also showed the same maximal response in vitro biological assays, and their dose–response curves paralled those of insulin. These findings were generally interpreted to indicate that the biological activity of the analogues was dependent only on binding to the insulin receptor and suggested that the region(s) of the insulin molecule associated with receptor binding and with the initiation of cellular processes were indistinguishable (Freychet et al., 1974; Gliemann & Gammeltoft, 1974; Cosmatos et al., 1978). We have recently reported (Burke et al., 1980) the divergence of the in vitro biological activity and receptor binding affinity of the synthetic analogue [21-asparaginamide-A]insulin. In stimulating glucose oxidation and lipogenesis, this analogue exhibited 12 and 14.8% activity, respectively, whereas in binding assays in rat liver membranes and isolated fat cells it showed binding affinities of 64 and 51%, respectively, compared to those of the natural hormone. A similar discrepancy between biological potency (4–5%) and

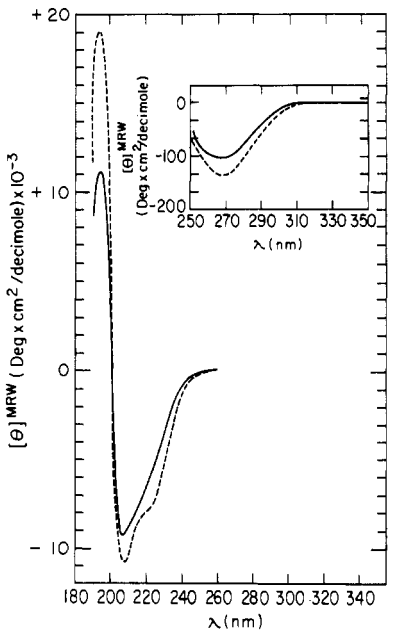

FIGURE 5: The near- and far-UV CD spectra of [Pro^{21}-B]insulin at 7.80×10^{-6} M (—) and zinc-free natural insulin at 7.00×10^{-6} M (---) in 0.001 N HCl.

receptor binding (25%) has been reported for porcupine (Horuk et al., 1980) and hagfish insulins (Emdin et al., 1977). The present investigation demonstrates that [Pro^{21}-B]insulin behaves differently from all other modified insulins. This analogue, in stimulating lipogenesis, exhibited 33% potency, while in binding studies to isolated fat cells and rat liver membranes, it showed 18% and 16% potency, respectively, relative to the natural hormone. The same maximal response was observed in the in vitro biological assays, and the dose–response curves paralleled those of insulin.

The present findings and previous data show that, with insulin analogues, no simple relationship exists between their receptor binding affinity and in vitro physiological potency, relative to that of insulin. In fact, the three a priori theoretical possibilities regarding this relationship have now been demonstrated by comparing the ratios of concentrations of analogue and insulin required for equivalent receptor binding and in vitro biological potency (experimentally evaluated at concentrations resulting in 50% of maximal inhibition of ^{125}I-insulin binding and 50% of maximal biological activity). The three possibilities are [insulin]/[analogue] (for binding) is equal to, greater than, or less than [insulin]/[analogue] (for biological activity).

In the case of most analogues, these concentration ratios are equal. For the [21-asparaginamide-A]insulin analogue, porcupine, and hagfish insulins, the binding ratio is smaller. For the present analogue, [Pro^{21}-B]insulin, the ratio of concentration for receptor binding is larger than the ratio for in vitro biological activity. This finding, in conjunction with the previous data, strongly suggests that within the insulin molecule two distinct regions are present: one primarily associated with

binding and the other with the expression of biological activity.

The similarity of the CD spectrum of the analogue and insulin at comparable concentrations and acidity (ca 7.0×10^{-6} M, 0.001 N HCl) indicates that for the most part there is little difference in their conformation. It is evident, however, that at this concentration, the molar ellipticities of insulin at each of its respective minima and maxima are greater than those of the analogue (Figure 5 and Table II). These differences are particularly evident at 193 and 274 nm. Further, at an insulin concentration of 1.74×10^{-5} M, the aforementioned differences in molar ellipticity are substantially greater (Table II). The magnitude of the molar ellipticity at 274 nm for insulin has been directly related to the β-pleated sheet conformation formed in the dimer by the interaction of the B^{22}–B^{29} segments of the monomers (Wood et al., 1975; Strickland & Mercola, 1976). These segments contain aromatic tyrosyl and phenylalanyl residues. However, insulin at a lower concentration (1.75×10^{-6} M) displays molar ellipticities comparable to those shown by the analogue at 7.8×10^{-6} M. At 1.75×10^{-6} M insulin, the concentration of dimer is negligible (Goldman & Carpenter, 1974; Wood et al., 1975). We suggest that the observed differences in molar ellipticity between insulin and the analogue (at 7.0×10^{-6} M) may be attributed, for the most part, to the presence of insulin dimer. The [Pro^{21}-B] analogue, it may be concluded, is monomeric at a concentration of 7.8×10^{-6} M, where insulin is in part dimeric. Whether the analogue would dimerize at substantially higher concentrations cannot be answered at this time.[2] The CD measurements, except perhaps for the difference in molar ellipticity at 193 nm, do not reveal any substantial difference in the monomeric form of insulin and analogues. Nevertheless, the behavior of the monomeric form(s) of insulin is distinctly different from that of [Pro^{21}-B]insulin, which indicates that the conformation(s) of these monomers is (are) not equivalent. It is of interest to note that in the crystalline two-Zn insulin hexamer the monomers assume two distinct conformations which differ from those observed in the four-Zn hexamer (Dodson et al., 1980). As pointed out previously, the insulin monomer shows a greater propensity to dimerize and to bind to the insulin receptor than does the analogue. This behavior suggests that the insulin monomer, depending on its immediate environment, may readily assume those conformation(s) favorable for dimerization and receptor binding. We do not imply that the conformation of the insulin monomer upon binding to its receptor is necessarily the same as that which insulin assumes in dimerization. In fact, it has been argued (Dodson et al., 1979) that some residues shown by X-ray studies to be involved in dimerization are unlikely to be involved in receptor binding. It appears that the analogue cannot assume conformations for binding equivalent to those of insulin, perhaps because the B^{21}-proline residue imposes a relative inflexibility on the molecule. The presumed greater probability for the B^{21}-proline analogue to maintain the β-bend in the B^{19}–B^{22} segment suggests that the B^{22}–B^{30} segment of the monomeric analogue is more constrained and differently fixed than in the insulin monomer and, further, that this constraint may displace other residues also involved in dimerization and binding.

We have observed that the biological activity of [Pro^{21}-B] insulin is greater than would be predicted on the basis of receptor binding. It may be that the very constraints imposed by the presence of proline in position B^{21} lead to a more favorable juxtaposition of those residues of hormone and receptor which interact to generate the biological response. We cannot at this time identify all those residues of the insulin molecule that are primarily involved in the expression of biological activity. Our previous results with [21-asparaginamide-A]-insulin implicate the free carboxyl group of asparagine-A^{21} in a region of the molecule that, independent of receptor binding, modulates biological activity. Insulin analogues in which the chemical modification does not seriously disturb this region would exhibit parallel and percentually equivalent changes in receptor binding potency and biological activity. This appears to be the case for the vast majority of insulin analogues examined to date. We suggest that the constraints placed on the molecule by the presence of proline in position B^{21} may result in the positioning of A^{21}-asparagine in the insulin receptor in such a way that the interaction between the analogue and the receptor is enhanced in comparison with that of the natural hormone, resulting in higher than predicted biological activity. It is of interest to note that in the crystal structure of insulin an interaction between Asn-A^{21} and Gly-B^{23} has been observed (Blundell et al., 1972).

We recognize that several models may be postulated to explain the relationships between binding affinity and in vitro biological activity of insulin analogues. All of the available data can be accommodated, with the least number of reasonable assumptions, by one of the two models which we have suggested previously (Burke et al., 1980). This model assumes that there is essentially a single class of insulin receptors which, when occupied, may result in biological response. These receptors are of the high-affinity type (Hollenberg & Cuatrecasas, 1976). The number of these receptors that need to be filled to elicit maximal biological activity is a small percentage of the total number of the high-affinity receptors ["spare-receptor" hypothesis, e.g., Kahn (1976)]. The number of these receptors that need be filled to achieve equivalent physiological response for insulin and an analogue depends on the intrinsic biological activity of the bound insulin or analogue. It is apparent from this model that the binding affinity of the hormone or analogue and its intrinsic biological activity need not be directly related. It may be concluded according to this model, that the intrinsic in vitro biological activity of a bound [Pro^{21}-B]insulin molecule is greater than that of a bound insulin molecule, albeit the affinity for the receptor is greater for insulin than for this analogue.

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Registry No. I, 86568-71-0; II, 86568-72-1; III, 86595-03-1; IV, 86568-73-2; IVa, 86595-04-2; bovine insulin A-chain–human [Pro^{21}]insulin B-chain, 86595-05-3; Boc-(N^{ω}-nitro)Arg-Gly-Phe-Phe-(Bzl)Tyr-Thr-Pro-(N^{ϵ}-benzyloxycarbonyl)Lys-(Bzl)Thr-OCH_2Ph, 52061-09-3; Boc-Pro, 15761-39-4; Boc-Leu-(Bzl)Tyr-Leu-Val-(*S*-diphenylmethyl)Cys-Gly, 52061-15-1; Boc-(Bzl)Ser-(N^{im}-tosyl)His-Leu-Val-(γ-benzyl)Glu-Ala, 52712-37-5; bovine insulin A-chain (reduced), 17289-65-5; Boc-Phe-Val-Asp(NH_2)-Glu(NH_2)-His-Leu-(*S*-diphenylmethyl)Cys-Gly, 52061-21-9.

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[2] The unusually low combination yield of A and B chains leading to the formation of the insulin analogue did not allow us to synthesize sufficient material to answer this question.

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Opioid Activities and Structures of α-Casein-Derived Exorphins†

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ABSTRACT: Exorphins, peptides with opioid activity, have previously been isolated from pepsin hydrolysates of α-casein [Zioudrou, C., Streaty, R. A., & Klee, W. A. (1979) *J. Biol. Chem. 254*, 2446–2449]. Analysis of these peptides shows that they correspond to the sequences 90–96, Arg-Tyr-Leu-Gly-Tyr-Leu-Glu, and 90–95, Arg-Tyr-Leu-Gly-Tyr-Leu, of α-casein. These peptides, as well as two of their analogues Tyr-Leu-Gly-Tyr-Leu-Glu (91–96) and Tyr-Leu-Gly-Tyr-Leu (91–95), have now been synthesized and characterized. Their opioid activity was examined by three different bioassays: (a) displacement of D-2-alanyl[*tyrosyl*-3,5-^{3}H]enkephalin-(5-L-methioninamide) and [^{3}H]dihydromorphine from rat brain membranes; (b) naloxone-reversible inhibition of adenylate cyclase in homogenates of neuroblastoma x glioma hybrid cells; (c) naloxonne-reversible inhibition of electrically stimulated contractions of the mouse vas deferens. The synthetic peptide of sequence 90–96 was the most potent opioid in all three bioassays and its potency was similar to that of the isolated α-casein exorphins. The synthetic peptides were totally resistant to hydrolysis by trypsin and homogenates of rat brain membranes, but were partially inactivated by chymotrypsin and subtilisin. The difference in opioid activity of α-casein exorphins may be related to differences in conformational flexibility observed by NMR spectroscopy.

Several families of opioid peptide have been found to occur naturally in the central nervous system. These include the pentapeptide enkephalins (Hughes et al., 1975), the endorphins which are larger (Bradbury et al., 1976; Guillemin et al., 1976; Li & Chung, 1976) and derived from a common precursor along with ACTH and α-MSH (Mains et al., 1977; Nakanishi et al., 1979), and the dynorphins (Goldstein et al., 1979; Kangawa et al., 1979), peptides of intermediate size, derived from yet another precursor (Kakidani et al., 1982). Each of these peptide groups shares the amino-terminal tetrapeptide sequence Tyr-Gly-Gly-Phe followed by Leu or Met. These pentapeptide sequences seem therefore to be the opiate recognition structure for all endogenous opioid peptides, collectively known as endorphins. The remainder of the endorphin sequence may serve to direct the peptide to the appropriate receptor type.

We and others have previously described the existence of peptides, in partial enzymatic digests of proteins derived from foodstuffs, which have opioid activities (Wajda et al., 1976; Klee et al., 1978; Zioudrou et al., 1979; Brantl et al., 1979;

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